ESTUARINE FISH 96-HOUR ACUTE TOXICITY
ECOLOGICAL EFFECTS BRANCH
STANDARD EVALUATION PROCEDURE

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Support Document 48

1. INTRODUCTION

A. When Required.

Acute toxicity studies with estuarine fish are required to support registration of an end-use product intended for direct application to the estuarine or marine environment. These studies are also necessary when it is expected that the pesticide would enter this environment in significant concentrations because of its expected use or mobility.

B. Purpose.

(1) To establish acute toxicity levels of the active ingredient to non-target marine and estuarine organisms;

(2) To compare toxicity information with measured or estimated pesticide residues in the estuarine or marine environment to assess potential impact to fish;

(3) To provide support for precautionary label statements to minimize adverse effects to estuarine or marine non-target organisms; and

(4) To indicate the need for further testing and/or field studies.

C. TEST MATERIAL

(1) Technical grade

Tests must be conducted with the technical grade of the active ingredient. If more than one active ingredient constitutes a technical product then the technical grade of each active ingredient must be tested separately.

(2) End Use Product

The applicant may be required to test the end-use product as well if:

(a) The end-use product will be introduced directly into the marine or estuarine environment when used as directed;

(b) The estuarine fish LC50 of the technical grade of the active ingredient is equal to or less than the expected environmental concentration in the marine or estuarine environment when the end-use product is used as directed;

(c) An ingredient of the end-use product is expected to enhance the toxicity of the end-use product beyond that expected from the active ingredient(s); or

(d) The technical product is not soluble in water but the formulated product is soluble in water. In that case the carriers or inerts of the formulated product must be tested in a solvent control.

2. MATERIALS AND METHODS: Standards/Data Acceptability for Estuarine Fish Study

A. Acceptable Protocols

EEB does not endorse any one protocol. It is sometimes necessary and desirable to alter the procedures presented in published protocols to meet the needs of the chemical or test organisms used. However, EEB does recommend some protocols as guidance for developing estuarine fish acute toxicity tests. These protocols include:

(1) Bahner, L. H., C.D. Craft, and D.R. Nimmo. 1975. A saltwater flow-through bioassay method with controlled temperature and salinity. Prog. Fish Cult. 37(3): 126-129.

(2) Committee on Methods for Toxicity Tests with Aquatic Organisms. 1975. Methods for acute toxicity tests with fish, macroinvertebrates and amphibians. U.S. Environmental Protection Agency, Ecol. Res. Series, EPA 660/375-009. 61 pp.

(3) Peltier, William. 1978. Methods for measuring the acute toxicity of effluents to aquatic organisms. U.S. Environmental Protection Agency, Ecol. Res. Series, EPA 600/4-78-012. 52 pp.

(4) Anonymous. 1978. Bioassay Procedures for the Ocean Disposal Permit Program. U.S. Environmental Protection Agency, Office of Research and Development. EPA-600/9-78-010. 121 pp.

(5) American Society for Testing and Materials. 1980. Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians. E 729-80. Published by ASTM Committee on Standards, 1916 Race Street, Philadelphia, PA 19103

These referenced protocols are presented as flexible guidance to help researchers design scientific protocols and to help the reviewer validate studies. It is important to recognize that fish tests are to be validated based on whether they provide scientifically sound information on the acute toxicity of the test material to estuarine fish that is useful in risk assessments and whether they fulfill guideline requirements. This is more important than whether they follow a referenced protocol step by step.

B. Test Organisms

(1) Acceptable species

The selected species should have a demonstrated sensitivity to known toxicants. If possible they should be species that occur in the area of exposure or be closely related to exposed species.

Several species are specifically referenced in "Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians" (ASTM, 1980) The preferred species are the silverside (Menidia sp.) and the sheepshead minnow (Cyprinodon variegatus). Other species may be acceptable if they are shown to be sensitive in acute toxicity tests.

(2) Size/Age/Physical Condition

Use of fish weighing between 0.5 and 5.0 grams is usually desireable. Very young (not actively feeding) should not be used, nor should sexually mature, spawning or recently spent fish. All fish should be from the same year class, and the standard length (tip of snout to end of caudal peduncle) of the longest fish should be no more than twice that of the shortest fish.

Test fish must be observed prior to testing for signs of disease, stress, physical damage and mortality. Injured, dead and abnormal individuals must be discarded. If the organisms show signs of disease or stress, remedial actions must be taken during the acclimation period and not during the test period. Organisms treated for disease may be used, but care should be taken to avoid using diseased animals. Organisms must not be used if they appear to be diseased on stressed or if more than 3% die during the 48 hours immediately prior to testing.

(3) Source/Acclimation

All organisms must be from the same source. This may include laboratory or commercial stocks. Fish captured in the wild are acceptable provided they meet the requirements pertaining to physical condition and age/size criteria mentioned above. Fish captured via gill netting, electro-shocking or chemical treatment must not be used.

After the fish have been received from the supplier or collected, they must be held for at least 7 days for observation and acclimation. To avoid unnecessary stress, rapid changes in water temperature and quality must be avoided. Feeding, handling and other maintenance must be such that the fish are not stressed, diseased or damaged. All organisms must be maintained under actual test conditions (temperature and water quality) for at least 48 hrs before the test actually begins. If used, young fish that have been actively feeding for less than 20 days must be fed up to the

beginning of the test. If they are larger (over 0.5 grams each) they should not be fed for 48 to 96 hours before the beginning of the test.

Feeding of the organisms should be limited to the time just prior to testing. This reduces the amount of organic material generated in the test containers. Organic matter may alter the test results by either adsorbing the chemical or increasing the oxygen demand of the test solution. Experience will dictate the exact "time pretest without feed" necessary and appropriate; it may vary between species. If the test material tends to bind to organic material, fish species that can survive at least 48 hours without feed before testing should be used. Pretest feeding information must be provided in the report.

C. Test Solution

(1) Source of Dilution Water

An adequate supply of dilution water that meets the minimum requirements mentioned below must be available. The water may be natural or reconstituted but must be able to support the test animals without stress, i.e. <5% mortality in 48-hour period pretest. Natural water must be of constant quality as described below.

Natural or reconstituted seawater of 30 to 34 °/oo salinity and pH of 8 to 8.3 should be used when testing marine (stenohaline) fish, and 10 to 17 °/oo salinity and pH 7.7 to 8.0 with estuarine (euryhaline) fish species. Natural seawater is considered to be 6 °/oo, and if the monthly range is less than 0.8 of a pH unit. See the guidance by the American Society for Testing Materials, 1980 or the Committee on Methods for Toxicity Tests with Aquatic Organisms, 1975 for guidance on specific amounts of minerals in reconstituted seawater. Commercial sources of seawater mixture are acceptable provided they do not adversely affect the test organism or alter the toxicity of the test material.

(2) Temperature

The recommended temperature for the silverside and the sheepshead minnow is 22'C. The temperature for other species should be determined on a case by case basis. The actual measured temperature should not deviate more the 1'C during the test. Greater deviations could affect test results.

D. Testing System

(1) Test Vessels

Test containers should be of welded stainless steel or glass.

If other materials such as polyethylene are used, the toxicant concentrations must be measured. For static tests fish (0.5 g each or more) are to be exposed in 19.6 liter containers with 15 liters of solution. Flow-through containers may be slightly larger.

(2) Photoperiod

A 16-hour light and 8-hour dark photoperiod should be provided, with a 15- to 30-minute transition period between light and dark.

(3) Loading

The loading factor or ratio of mass of test organisms to volume of test solution must not be high enough to affect test results. The size of the test container should be such that the loading factor (test organism mass per volume of test solution) is no greater than 0.8 g/L of test solution in static tests at or below 17'C or 0.5 g/L of test solution at higher temperatures. For flow-through tests, the loading should be no greater than 1 g/L of test solution passing through the chamber in 24 h, and must not exceed 10 g/L of test solution at any time at or below 17'C or 5 g/L of test solution at higher temperatures.

(4) Solvents

If solvents other than water are necessary, they should be used sparingly, not to exceed 0.5 ml/l in any static test solution, and 0.1 ml/l in flow-through system. If solvents are necessary, the following are acceptable:

dimethyl formamide
triethylene glycol
methanol
acetone
ethanol

(5) Flow Through Tests

Flow-through tests are not usually required. They may be necessary if the test chemical is relatively insoluble, has a short halflife or is volatile. The metering system chosen for a flowthrough study must reproducibly supply appropriate toxicant concentrations at a consistent flow rate. Metering systems should be calibrated before and after each study and checked twice daily during the test period. Flow rates should be 5 to 10 volume additions per 24 hours. Flow-through systems should be constructed so that the organisms are not stressed by turbulence. Concentrations must be measured in a flow-through system to verify the metering system.

E. Test Design

(1) Test Levels (nominal/measured)

Initially, range-finding tests may be necessary to define concentrations of the toxicant needed for definitive studies. Test reports should provide information describing range-finding study procedures and results. The information should include sample sizes, dosage levels, and mortality data.

If it can be shown (by testing at least 30 individuals) that a chemical will have an LC50 greater than 100 ppm, a definitive study need not be performed.

Definitive acute toxicity tests normally are designed to include one or more control groups and a geometric series of at least 5 toxicant concentrations to be tested. Each designated treatment group should be exposed to a concentration of toxicant that is at least 60% of the next highest concentration. If a formulated product is tested, it should be clearly stated in the test report whether results are expressed in terms of active ingredient alone or as total formulated product.

(2) Number of Test Animals

The test vessels must contain no less than 10 organisms per level in a static test and no less than 20 organisms per level in a flow-through design. These 10 or 20 organisms may be in one container or may be divided between 2 or more replicate containers. In every case, they must be randomly assigned to the test containers.

(3) Controls

Each test requires a concurrent control using the same dilution water and same number of organisms at each test level. If any solvent other than water is used a solvent control should also be conducted. This solvent control should contain the highest concentration of the solvent that was added to any of the test chambers. A test is not acceptable if more than 10% of the control organisms die during a test.

(4) Beginning the Test

Static acute tests are begun either by adding the test material to the test chambers after the organisms have been added or by adding the organisms to the test chambers within 30 minutes after the test material has been added to the dilution water.

(5) Measurement of Diluent Characteristics

Temperature should be measured hourly throughout the acclimation and test period in at least one test chamber if the test containers are not in a temperature controlled water bath because air temperature may change more frequently and to a greater extent than water, thus affecting the test container temperature. If the temperature is controlled by a water bath, the temperature of the bath may be recorded every 6 hours. Temperature should not vary more than one degree during entire study period

The dissolved oxygen (DO) concentration must be measured at the beginning of the test and every 48 hours thereafter to the end of the test in the control and the high, medium, and low concentration as long as fish are present at those concentrations. The DO level during the first 48 hours must be between 60% and 100% of saturation and between 40% and 100% saturation after 48 hours. In the flow-through test, the DO concentrations in each chamber must be between 60% and 100% saturation at all times during the study.

The pH should be measured at the beginning and end of the test in the control and the high, medium, and low toxicant concentrations.

(6) Chemical Analysis

It may be preferred to chemically analyze test solutions to determine exact concentrations of pesticides. It is particularly important that residues are measured if:

(a) The test solutions were aerated;
(b) The test material was volatile, insoluble or precipitated out of solution;
(c) The test containers were not made of glass or stainless steel; (d) The test chemical is known to adsorb to the test containers sturctural material; or
(e) A flow through system is used (measurement verifies accuracy of metering system).

3. REPORTING REQUIREMENTS

The test report submitted to the Agency must fully describe the materials and methods used to perform the study. The reviewer must be able to determine from the report that the study was performed under conditions that render the results acceptable for use in a risk assessment and or for fulfilling a guideline requirement. The following information is particularly important for a complete evaluation.

A. Test Material

If the study is to be performed with the technical grade product, the test material should be clearly identified as to source, batch, and exact purity. Simply identifying the material as technical may not be suffcient because the per cent active ingredient of some newer products may increase with time as the manufacturing process is improved to produce greater purity.

For studies involving the end use product, the exact per cent of the active ingredient and the type of formulation (e. g. granular, wettable powder) of the test material should be described. It should clearly state in the test report whether the results are expressed in terms of active ingredient or as formulation.

B. Dilution Water

Test reports submitted to the Agency should include a complete description of dilution waters used in the test. Descriptions should include identification of the source, the chemical characteristics of the water, and information on any pretreatment.

C. Holding of Test Organisms

Test reports should include complete information on holding and acclimation conditions including feeding schedules and treatment for diseases.

D. Mortality/Observable Effects

The criteria for determining effects must be defined. The raw data or percentage of deaths at each level as well as the number of organisms tested per level must be reported for each 24-hour period. Toxic symptoms (physical and behavioral) should be described.

E. Calculated LC50

The statistically calculated LC50 with 95% confidence limits and the method of calculation must be presented. In lieu of a calculated LC50, the study may show that the LC50 is greater than 100 ppm. The slope of the dose-response line should be calculated and reported. Some reports have a graphic (log probit/dose concentration) presentation which is helpful.

F. Temperature/DO/pH

Dissolved oxygen, pH measurements and the range and average temperature during the study should be reported.

G. Chemical Analysis

If chemical analyses were conducted, the test report should provide information on the methods (references) utilized and the results of analyses. Residues found at the beginning and end of the study should be reported.

Variability in Measured Concentrations

The goal for limiting variability of measurements between replicates of the same concentration, and over time in the same concentration, is maintaining the ratio of the highest concentration at 1.5 or less. A test may be rejected if it exceeds this amount.

An important factor in considering the limits of variability is the avoidance of overlapping mean test concentrations between test levels. High variability puts into question the reliability of the environmental chemistry method and/or the concentrations on which to base statistical analysis and toxicological conclusions. If variability beyond the 1.5 ratio occurs, an exception to it should be justified.

This justification should clearly state the problem, explain why it occurred, provide scientific justification, and identify all measures taken to mitigate the problem. The justification should also include the fully developed chemistry method, including the documentation necessary for a bench chemist to review and evaluate it.

For cases in which variability problems are suspected, registrants wishing to avoid possible rejection of a study are strongly advised to conduct extensive preliminary trials. If it becomes clear that high variability cannot be avoided an exception should be justified.

It is recommended that any justification be provided in advance. EFED scientists will decide on the validity of the rationale for the exception, and possibly recommend other methods to reduce potential variability.

H. Testing Protocols

The test report should include reference to the testing protocol(s) used during the study.

3. REVIEWERS EVALUATION

A. Review of Test Conditions

The reviewer should identify each aspect of the reported procedure that is inconsistent with recommended protocol. The significance of these deviations must be determined. The

number of deviations and their severity will determine the validity of the study and the interpretation of the results.

B. Verification of Statistical Analyses

The reviewer should ensure that the LC50 has been properly derived by recalculating these values. An acceptable acute toxicity test should provide not only an LC50 but also a NOEL (no observed effect level) and a slope of the dose/mortality response. The NOEL is the highest concentration at which no mortality or other toxic signs occurred. These data can give further insight into the toxicological characteristics of the chemical such as whether the response is gradual, over a wide range or rapid.

If the recalculated results differ substantially from the submitted results the reviewer should note this and attempt to explain the differences.

C. Conclusions

(1) Categorization of Results

The significance of inconsistencies in the test procedures must be determined by the reviewer so that the results of the test can be categorized as to whether they fulfill Part 158 regulations and are useful in performing a risk assessment. Categories are described as:

a. Core: All essential information was reported and the study was performed according to recommended protocols. Minor inconsistencies with standard methodologies may be apparent, however the deviations do not detract from the study's soundness or intent. Studies within this category fulfill the basic requirements of current guidelines and are acceptable for use in a risk assessment.

b. Supplemental: Studies in this category are scientifically sound, however they were performed under conditions that deviated substantially from recommended protocols. Results do not meet guideline requirements, however the information may be useful in a risk assessment.

Some of the conditions that may place a study in a supplemental category include:

1. Unacceptable test species;
2. Inappropriate test material;
3. Concentrations tested were less than 100 ppm but not high enough to produce an effect on the organisms or a precise LC50; and
4. Deviations from recommended test solution characteristics (Variations in DO, temperature,

hardness, and pH can affect toxicological response).

c. Invalid: These studies provide no useful information. They may be scientifically unsound, or they were performed under conditions that deviated so significantly from recommended protocols that the results will not be useful in a risk assessment.

Examples of studies placed in this category commonly include those where the test system was aerated, test vessels were constructed from materials other than glass, or there were problems of solubility or volatility of the test material. Unless acceptable chemical analyses of actual toxicant concentrations were performed in studies such as these, the reviewer cannot be sure that test organisms were actually exposed to nominally designated concentrations.

A study where the test material was not properly identified can also be invalidated.

(2) Rationale

Identify what makes the study supplemental or invalid. While all deviations from recommended protocol should be noted, the reviewer is expected to exercise judgement in the area of study categorization.

(3) Repairability

Indicate whether the study may be upgraded or given a higher validation category if certain conditions are met. Usually this would involve the registrant submitting more data about the study.

D. Descriptive Classification

The reviewer should indicate what the results were and how much information can be drawn from them. At a minimum, an acute toxicity test will provide an LC50 with 95% confidence limits. This should allow classifying the test material based on the following scheme:

LC50 (ppm)	Category Description
< 0.1	very highly toxic
0.1 - 1	highly toxic
1 - 10	moderately toxic
10 - 100	slightly toxic
>100	practically non-toxic

These descriptive categories are for inter-chemical comparison only and do not reflect actual environmental risk to the test organism. The results may provide other useful information such as slope or a no observed effect level (NOEL). These additional data are useful in a risk assessment.

E. References

The reviewer should reference any information used in the validation procedure. This should include protocol documents, statistical methods, or information taken from files of other branches.